DESCRIPTION

SYSTEM FOR EXPRESSING HYPERTHERMOSTABLE PROTEIN

Technical Field

The present invention relates to a hyperthermostable protease useful as an enzyme for industrial use, a gene encoding the same and a method of producing the enzyme by genetic engineering technique.

Background Art

A protease is an enzyme that cleaves peptide bonds in proteins. A number of such enzymes have been found in animals, plants and microorganisms. The protease is used as a reagent for laboratory use and as a pharmaceutical, as well as in industrial fields, for example, as an additive for a detergent, for processing foods and for chemical synthesis utilizing a reverse reaction. Therefore, it can be said that the protease is an extremely important enzyme for industries. Since high physical and chemical stability is required for a protease used in industrial fields, a thermostable enzyme is preferably used among others. Since proteases produced by bacteria of genus *Bacillus* exhibit relatively high thermostability, they are mainly used as proteases for industrial use. However, in search of a more

superior enzyme, attempts have been made to obtain an enzyme from a microorganism growing at high temperature, for example, a thermophilic bacterium of genus *Bacillus* or a hyperthermophile.

For example, a hyperthermophile *Pyrococcus furiosus* is known to produce a protease (Appl. Environ. Microbiol., 56:1992-1998 (1990); FEMS Microbiol. Letters, 71:17-20 (1990); J. Gen. Microbiol., 137:1193-1199 (1991)).

In addition, a hyperthermophile, *Pyrococcus* sp. strain KOD1, is reported to produce a thiol protease (a cysteine protease) (Appl. Environ. Microbiol., 60:4559-4566 (1994)). Hyperthermophiles of genus *Thermococcus*, genus *Staphylothermus* and genus *Thermobacteroides* are also known to produce proteases (Appl. Microbiol. Biotechnol., 34:715-719 (1991)).

The proteases from the hyperthermophiles as described above have high thermostability. Therefore, it is expected that they may be used in place of the thermostable proteases currently in use or in a field in which use of a protease has not been considered.

However, most of the microorganisms producing these enzymes grow only at high temperature. For example, *Pyrococcus furiosus* needs to be cultured at 90-100°C. Culturing at such high temperature is disadvantageous in view of energy cost. Furthermore, the productivities of the

proteases from the hyperthermophiles are lower than the productivities of the conventional microbial proteases. Thus, the methods for industrially producing the proteases from the hyperthermophiles have problems.

By the way, production of an enzyme by genetic engineering technique by isolating the gene for the enzyme of interest and introducing it into a host microorganism that can readily be cultured is currently common in the art. However, the gene for the enzyme introduced into the host is not always expressed so efficiently as expected. It is believed that the main cause is that the GC content or the codon usage of the introduced gene is different from those of the genes of the host. Therefore, it is necessary to optimize the expression method for each gene to be introduced and/or each host in order to accomplish a suitable productivity of an enzyme for the intended use.

Objects of the Invention

The objects of the present invention are to provide a protease from a hyperthermophile which is advantageous for industrial use, to isolate a gene encoding the protease from the hyperthermophile, and to provide a method of producing the hyperthermostable protease using the gene by genetic engineering technique in order to solve the problems as described above.

Summary of the Invention

Among proteases produced by hyperthermophiles, some may be classified into the subtilisin-type of alkaline proteases based on the amino acid sequence homology. When a gene for such a protease is introduced into *Bacillus subtilis* which is generally used for production by genetic engineering technique, the productivity of this enzyme is much less than that of a protein inherently produced by *Bacillus subtilis*.

The present inventors have studied intensively and found that, by placing a gene encoding a signal peptide (signal sequence) derived from a subtilisin upstream a protease gene derived from a hyperthermophile to be expressed, and modifying the amino acid sequence around the cleavage site, the gene of interest is expressed in *Bacillus subtilis* with high efficiency. Furthermore, it has been found that the expression level of the enzyme can be increased by deleting a portion that is not essential for the enzymatic activity in the protease gene derived from the hyperthermophile of interest. Thus, the present invention has been completed.

The present invention is outlined as follows. The first invention of the present invention is a thermostable protease having an amino acid sequence represented by the

SEQ ID NO:1 of the Sequence Listing, and a protease having an amino acid sequence in which one or several amino acid residues are deleted, substituted, inserted or added in the amino acid sequence represented by the SEQ ID NO:1 of the Sequence Listing and having a thermostable protease activity.

The second invention of the present invention is a gene encoding the thermostable protease of the first invention, and a thermostable protease gene that hybridizes with the gene.

The third invention of the present invention is a gene to be used for producing a thermostable protease derived from a hyperthermophile by genetic engineering technique, characterized in that the gene encodes an amino acid sequence represented by formula I:

SIG-Ala-Gly-Gly-Asn-PRO [I]

wherein SIG represents an amino acid sequence of a signal peptide derived from a subtilisin, PRO represents an amino acid sequence of a protein to be expressed. Preferably, SIG is the amino acid sequence represented by the SEQ ID NO:3 of the Sequence Listing. Preferably, PRO is an amino acid sequence of a hyperthermostable protease derived from a hyperthermophile, more preferably, an amino acid sequence of a protease derived from *Pyrococcus furiosus*.

The fourth invention of the present invention

relates to a method of producing a protein by genetic engineering technique, characterized in that the method comprises culturing a bacterium of genus *Bacillus* into which the gene of the third invention is introduced, and collecting the protein of interest from the culture.

The fifth invention of the present invention is a plasmid used for producing a protein by genetic engineering technique, characterized in that the gene of the third invention is inserted into the plasmid.

A mutation such as deletion, substitution, insertion or addition of one to several amino acid residues in an amino acid sequence may be generated in a naturally occurring protein including the protein disclosed by the present invention. Such mutation may be generated due to a polymorphism or a mutation of the gene encoding the protein, or due to a modification of the protein in vivo or during purification after synthesis may occur. Nevertheless, it is known that such a mutated protein may exhibit physiological and biological activities equivalent with those of a protein without a mutation. This is applicable to a protein in which such a mutation is introduced into its amino sequence artificially, in which case it is possible to generate a wide variety of mutations. For example, it is known that a polypeptide in which a cysteine residue in the amino acid sequence of human interleukin-2 (IL-2) is

substituted with a serine residue retains an interleukin-2 activity (Science, 224:1431 (1984)). Thus, a protease having an amino acid sequence in which one or several amino acid residues are deleted, substituted, inserted or added in the amino acid sequence disclosed by the present invention and having a protease activity equivalent with that of the protease of the present invention is within the scope of the present invention.

As used herein, "a gene which hybridizes (with a particular gene)" is a gene having a base sequence similar to that of the particular gene. It is likely that a gene having a base sequence similar to that of a particular gene encodes a protein having an amino acid sequence and a function similar to those of the protein encoded by the particular gene. Similarity of base sequences of genes can be examined by determining whether or not the genes or portions thereof form a hybrid (hybridize) each other under stringent conditions. By utilizing this procedure, a gene that encodes a protein having a similar function with that of the protein encoded by the particular gene can be obtained. That is, a gene having a similar base sequence with that of the gene of the present invention can be obtained by using the gene obtained by the present invention or a portion thereof as a probe to carry out hybridization according to a known method. Hybridization

can be carried out according to the method, for example, as described in T. Maniatis et al. eds., Molecular Cloning: A Laboratory Manual 2nd ed., published by Cold Spring Harbor Laboratory, 1989. More specifically, hybridization can be carried out under the following conditions. Briefly, a membrane onto which DNAs are immobilized is incubated in 6xSSC (1xSSC represents 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrorridone, 0.1% Ficoll 400, 0.01% denatured salmon sperm DNA at 50°C for 12-20 hours with a probe. After incubation, the membrane is washed until the signals for the immobilized DNAs can be distinguished from background, starting from washing in 2xSSC containing 0.5% SDS at 37°C while decreasing the SSC concentration down to 0.1x and raising the temperature up to 50°C.

Alternatively, instead of hybridization, a gene amplification method (e.g., PCR method) which employs portions of the base sequence of the gene obtained by the present invention as primers can be utilized. Whether or not the gene thus obtained encodes a protein having the function of interest can be determined by expressing the gene utilizing a suitable host and a suitable expression system and examining the activity of the resulting protein.

Brief Description of the Drawings

Figure 1 is the restriction enzyme map of the plasmid pSTC3.

Figure 2 compares the amino acid sequences of Protease PFUS, Protease TCES and a subtilisin.

Figure 3 compares the amino acid sequences of Protease PFUS, Protease TCES and a subtilisin.

Figure 4 compares the amino acid sequences of Protease PFUS, Protease TCES and a subtilisin.

Figure 5 compares the amino acid sequences of Protease PFUS, Protease TCES and a subtilisin.

Figure 6 is the restriction enzyme map of the plasmid pSNP1.

Figure 7 is the restriction enzyme map of the plasmid pPS1.

Figure 8 is the restriction enzyme map of the plasmid pNAPS1.

Detailed Description of the Invention

The hyperthermostable protease according to the present invention includes proteases from various hyperthermophiles. For example, WO 95/34645 describes proteases from *Pyrococcus furiosus* and *Thermococcus celer*.

A protease gene from *Pyrococcus furiosus* DSM3638 was isolated from a genomic DNA library of the strain based on the expression of a thermostable protease activity. A

plasmid containing this gene is designated as the plasmid pTPR12. *Escherichia coli* JM109 transformed with this plasmid is designated and indicated as *Escherichia coli* JM109/pTPR12, and deposited on May 24, 1994 (the date of the original deposit) under Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan under accession number FERM BP-5103.

This protease is designated as Protease PFUL hereinafter. Protease PFUL is a protease having high thermostability and exhibits a protease activity even at 95°C.

The base sequence of the DNA fragment derived from *Pyrococcus furiosus* inserted into the plasmid pTPR12 has been determined. The base sequence of the portion of approximately 4.8 kb bordered by two DraI sites in the DNA fragment inserted into the plasmid pTPR12 is shown in the SEQ ID NO:5 of the Sequence Listing. Furthermore, the amino acid sequence of the gene product deduced from the base sequence is shown in the SEQ ID NO:6 of the Sequence Listing. In other words, the amino acid sequence as shown in the SEQ ID NO:6 of the Sequence Listing is the amino acid sequence of Protease PFUL. As shown in the sequence,

Protease PFUL consists of 1398 amino acid residues and is a protease with a high molecular weight of over 150,000.

Comparison of the amino acid sequence of Protease PFUL as shown in SEQ ID NO:6 of the Sequence Listing with known amino acid sequences of proteases from microorganisms has revealed that the amino acid sequence of the first half portion of Protease PFUL is homologous to those of a series of alkaline serine proteases represented by a subtilisin (Protein Engineering, 4:719-737 (1991)), and that there is extremely high homology around the four amino acid residues which are believed to be important for the catalytic activity of the protease.

As described above, it has been found that a region common among proteases derived from mesophiles is conserved in the amino acid sequence of Protease PFUL produced by a hyperthermophile *Pyrococcus furiosus*. Thus, it is expected that a homologous protease produced by a hyperthermophile other than *Pyrococcus furiosus* also has this region.

For example, a gene for a hyperthermostable protease can be screened by performing PCR using a chromosomal DNA from various hyperthermophiles as a template and the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R in combination as primers. These oligonucleotides are synthesized based on the base sequence

in the Protease PFUL gene which encodes a region exhibiting high homology with subtilisins or the like within the amino acid sequence of Protease PFUL. The base sequences of oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R are shown in the SEQ ID NOS:7, 8, 9 and 10 of the Sequence Listing, respectively.

As a hyperthermophile from which the protease according to the present invention is derived, a bacterium belonging to genus *Pyrococcus*, genus *Thermococcus*, genus *Staphylothermus*, genus *Thermobacteroides* and the like can be used. As a bacterium belonging to genus *Thermococcus*, for example, *Thermococcus celer* DSM2476 can be used. This strain is available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. When performing PCR using a chromosomal DNA from *Thermococcus celer* DSM2476 as a template and a combination of the oligonucleotides PRO-1F and PRO-2R or the oligonucleotide PRO-2F and Pro-4R as primers, specific DNA fragments are amplified, indicating the presence of a protease gene. Furthermore, by creating recombinant plasmids in which the DNA fragments are inserted into an appropriate plasmid vector and determining the base sequences of the inserted DNA fragments by dideoxy method, the amino acid sequences encoded by the fragments can be deduced. As a result, it proved that such DNA fragments encode an amino acid sequence that is homologous

to the amino acid sequences of Protease PFUL and alkaline serine proteases from various microorganisms and that the PCR-amplified DNA fragments were amplified from a protease gene as a template.

Next, a gene for a hyperthermostable protease (for example, a gene for a hyperthermostable protease produced by *Thermococcus celer*) can be obtained by screening a gene library from a hyperthermophile using the PCR-amplified DNA fragment or the oligonucleotide as described above as a probe.

For example, a phage clone containing the gene of interest can be obtained by performing plaque hybridization against a library using the PCR-amplified DNA fragment as a probe. Such library is generated by ligating lambda GEM-11 vector (Promega) and DNA fragments resulting from partial digestion of the chromosomal DNA from *Thermococcus celer* DSM2476 with a restriction enzyme Sau3AI, then packaging them into lambda phage particles by in vitro packaging method.

It is found that a protease gene exists in a SacI fragment of approximately 1.9 kb by analyzing a DNA fragment contained in a phage clone thus obtained. Furthermore, it is found that this fragment lacks the 5' region of the protease gene by determining its base sequence. The 5' region can be obtained by PCR using a

cassette and cassette primers (Takara Shuzo Gene Technology Product Guide, 1994-1995, pp.250-251). Thus, a DNA fragment which covers the 5' region of the hyperthermostable protease gene which is absent in the plasmid pTCS6 can be obtained. Furthermore, the base sequence of the entire hyperthermostable protease gene derived from *Thermococcus celer* can be determined from the base sequences of the two DNA fragments.

The base sequence of an open reading frame found in the determined base sequence is shown in the SEQ ID NO:11 of the Sequence Listing, and the amino acid sequence deduced from the base sequence is shown in the SEQ ID NO:12 of the Sequence Listing. The base sequence of the gene encoding the hyperthermostable protease from *Thermococcus celer* and the amino acid sequence of the protease were thus determined. This protease is designated as Protease TCES.

An expression vector in which the entire Protease TCES gene is reconstituted by combining the two DNA fragments can be constructed. However, when using *Escherichia coli* as a host, a transformant into which the expression plasmid of interest had been introduced was not obtained, probably because the generation of the product expressed from the gene in cells may be harmful or lethal to *Escherichia coli*. In such a case, for example, it is possible to use *Bacillus subtilis* as a host for

extracellular secretion of the protease and to determine the activity.

As a *Bacillus subtilis* strain, *Bacillus subtilis* DB104 can be used, which is a known strain as described in Gene, 83:215-233 (1989). As a cloning vector, the plasmid pUB18-P43 can be used, which is a generous gift from Dr. Sui-Lam Wong, University of Calgary. The plasmid contains a kanamycin-resistance gene as a selectable marker.

A recombinant plasmid in which the Protease TCES gene is inserted downstream the P43 promoter in the plasmid vector pUB18-P43 is designated as the plasmid pSTC3. *Bacillus subtilis* DB104 transformed with this plasmid is designated and indicated as *Bacillus subtilis* DB104/pSTC3, and was deposited on December 1, 1995 (the date of the original deposit) under Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan under accession number FERM BP-5635.

The restriction enzyme map of the plasmid pSTC3 is shown in Figure 1. In Figure 1, the bold line indicates the DNA fragment inserted into the plasmid vector pUB18-P43.

A thermostable protease activity is found in either of the culture supernatant and the cell extract of

the culture of *Bacillus subtilis* DB104/pSTC3.

Main properties of a crude enzyme preparation of the protease obtained from the culture of the transformant are as follows.

(1) Action:

Degrades casein and gelatin to generate short chain polypeptides.

Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) to generate a fluorescent substance (7-amino-4-methylcoumarin).

Hydrolyzes succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) to generate a yellow substance (p-nitroaniline).

(2) Optimal temperature:

Exhibits an enzymatic activity at 37-95°C, with the optimal temperature being 70-80°C.

(3) Optimal pH:

Exhibits an enzymatic activity at pH 5.5-9, with the optimal pH being pH 7-8.

(4) Thermostability:

Retains 90% or more of its enzymatic activity after treatment at 80°C for 3 hours.

When aligning the amino acid sequences of Protease PFUL, Protease TCES and a subtilisin (subtilisin

BNP'; Nucl. Acids Res., 11:7911-7925 (1983)) such that homologous regions match each other as shown in Figures 2-5, it is found that, at the C-terminus and between the homologous regions of Protease PFUL, there are sequences which are not found in Protease TCES or the subtilisin. From these results, a protease having a molecular weight lower than that of Protease PFUL and similar to Protease TCES or subtilisins may exist in *Pyrococcus furiosus* in addition to Protease PFUL.

Thereupon, Southern hybridization against a chromosomal DNA prepared from *Pyrococcus furiosus* was carried out using a DNA probe from the homologous region, and a signal other than that for the Protease PFUL gene was observed, indicating the existence of another protease gene.

This novel protease gene can be isolated by the following procedure.

For example, a DNA fragment containing a gene encoding the novel protease is obtained by digesting a chromosomal DNA from *Pyrococcus furiosus* with an appropriate restriction enzyme and performing Southern hybridization against the digested DNA as described above. The base sequence of the DNA fragment is determined to confirm that the base sequence encodes an amino acid sequence homologous to the above-mentioned protease. If the DNA fragment does not contain the entire gene of interest,

1000054-030502

the remaining portion is further obtained by inverse PCR method or the like.

For example, when a chromosomal DNA from *Pyrococcus furiosus* is digested with restriction enzymes SacI and SpeI (Takara Shuzo) and is used for Southern hybridization, a signal of approximately 0.6 kb in size is observed. DNA fragments of this size are recovered, inserted between the SpeI-SacI sites in the plasmid vector pBluescript SK(-) (Stratagene), and *Escherichia coli* JM 109 is transformed with the resulting recombinant plasmids. A clone into which the fragment of interest is incorporated can be obtained from the transformants by colony hybridization using the same probe as that used for the Southern hybridization as described above. Whether or not the plasmid harbored by the obtained clone has the sequence that encodes the protease can be confirmed by determining the base sequence of the DNA fragment inserted into the plasmid. The presence of the protease gene in the plasmid was thus confirmed. This plasmid is designated as the plasmid pSS3.

It is found that the amino acid sequence deduced from the base sequence of the DNA fragment inserted into the plasmid pSS3 has homology with sequences of subtilisins, Protease PFUL, Protease TCES and the like. The product of the protease gene distinct from the Protease PFUL gene, a

portion of which was newly obtained from *Pyrococcus furiosus* as described above, is designated as Protease PFUS. The regions which encode the N-terminal and C-terminal regions of the protease can be obtained by inverse PCR method.

Primers used for inverse PCR can be prepared based on the base sequence of the DNA fragment inserted into the plasmid pSS3. A chromosomal DNA from *Pyrococcus furiosus* is digested with an appropriate restriction enzyme, and the resulting DNA fragments are then subjected to an intramolecular ligation reaction. By performing PCR using the reaction mixture as a template and the above-mentioned primers, DNA fragments corresponding to the regions flanking the fragment for the protease gene contained in the plasmid pSS3 can be obtained. The amino acid sequence of the enzyme protein encoded by these regions can be deduced by analyzing the base sequences of the DNA fragments thus obtained. Furthermore, primers capable of amplifying the entire Protease PFUS gene using a chromosomal DNA from *Pyrococcus furiosus* as a template can be prepared. The primers NPF-4 and NPR-4 can be designed. The primer NPF-4 has the base sequence immediately upstream the initiation codon of the Protease PFUS gene and can introduce a BamHI site 5' to the sequence. The primer NPR-4 has a sequence complementary to the 3' portion of the

Protease PFUS gene and can introduce a SphI site 5' to the sequence.

The base sequences of the primers NPF-4 and NPR-4 are shown in the SEQ ID NOS:13 and 14 of the Sequence Listing. These two primers can be used to amplify the entire Protease PFUS gene using a chromosomal DNA from *Pyrococcus furiosus* as a template.

Like Protease TCES, Protease PFUS can be expressed in *Bacillus subtilis* as a host. A plasmid for expressing Protease PFUS can be constructed based on the expression plasmid for Protease TCES, pSTC3. Specifically, a plasmid for expressing Protease PFUS can be constructed by replacing the Protease TCES gene in the plasmid pSTC3 with the DNA fragment containing the entire Protease PFUS gene amplified by PCR with the primers as described above. The expression plasmid thus constructed is designated as the plasmid pSNP1. *Bacillus subtilis* DB104 transformed with this plasmid is designated and indicated as *Bacillus subtilis* DB104/pSNP1, and was deposited on December 1, 1995 (the date of the original deposit) under Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan under accession number FERM BP-5634. The restriction enzyme map of the plasmid pSNP1 is

shown in Figure 6.

The base sequence corresponding to an open reading frame in the gene encoding Protease PFUS and the amino acid sequence of Protease PFUS deduced from the base sequence are shown in the SEQ ID NOS: 15 and 16 of the Sequence Listing, respectively.

A thermostable protease activity is found in either of the culture supernatant and the cell extract from the culture of *Bacillus subtilis* DB104/pSNP1. That is, a portion of the expressed Protease PFUS is secreted into the culture supernatant.

Main properties of the protease obtained from the culture of the transformant are as follows.

(1) Action:

Degrades casein and gelatin to generate short chain polypeptides.

Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) to generate a fluorescent substance (7-amino-4-methylcoumarin).

Hydrolyzes succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) to generate a yellow substance (p-nitroaniline).

(2) Optimal temperature:

Exhibits an enzymatic activity at 40-110°C, with

the optimal temperature being 80-95°C.

(3) Optimal pH:

Exhibits an enzymatic activity at pH 5-10, with the optimal pH being pH 6-8.

(4) Thermostability:

Retains 90% or more of its enzymatic activity after treatment at 95°C for 8 hours.

(5) pH stability

Retains 95% or more of its activity after treatment at pH 5-11 at 95°C for 60 minutes.

(6) Molecular weight

Exhibits a molecular weight of approximately 45 kDa on SDS-PAGE.

Protease genes homologous to the Protease TCES gene and the Protease PFUS gene can be obtained from hyperthermophiles other than *Pyrococcus furiosus* and *Thermococcus celer* using a method similar to that used to obtain the Protease TCES gene and the Protease PFUS gene.

A DNA fragment of approximately 1 kb which encodes a sequence from the residue at position 323 to the residue at position 650 of the amino acid sequence of Protease PFUL as shown in the SEQ ID NO:6 of the Sequence Listing can be prepared and used as a probe for genomic Southern hybridization against chromosomal DNAs from *Staphylothermus marinus* DSM3639 and *Thermobacteroides*

proteoliticus DSM 5265. As a result, signals are observed at the position of approximately 4.8 kb for the chromosomal DNA from *Staphylothermus marinus* digested with PstI (Takara Shuzo) and at the position of approximately 3.5 kb for the chromosomal DNA from *Thermobacteroides proteoliticus* digested with XbaI.

From these results, it proved that there are sequences homologous to those of the genes for Protease PFUL, Protease PFUS and Protease TCES and the like on the chromosomal DNAs from *Staphylothermus marinus* and *Thermobacteroides proteoliticus*. The genes encoding the hyperthermostable proteases in *Staphylothermus marinus* and *Thermobacteroides proteoliticus* can be isolated and identified from the DNA fragments thus detected by using a method similar to that used to isolate and identify the genes encoding Protease TCES and Protease PFUS.

In general, it is believed that use of a promoter that acts effectively in a host rather than a promoter that is inherently associated with the gene encoding the protein of interest would be advantageous in order to prepare a protein in a large quantity by genetic engineering technique. Although the P43 promoter used to construct the expression systems for Protease TCES and Protease PFUS is a promoter derived from *Bacillus subtilis*, it was not sufficiently effective to express the two proteases.

Thereupon, a gene that is expressed at high level in *Bacillus subtilis*, particularly a gene for a secreted protein, may be utilized in order to increase the expression level. Genes for α-amylase or various extracellular proteases can be used. For example, it is expected that use of a promoter and a signal peptide-encoding region of a subtilisin gene may increase the expression level of Protease PFUS.

Specifically, Protease PFUS can be expressed as a fused protein under control of the promoter of the subtilisin gene by placing the entire Protease PFUS gene downstream the region encoding the signal peptide of the subtilisin gene including the promoter region such that the translational frames of the two genes match each other.

For example, the gene encoding subtilisin E can be used as the subtilisin gene used in the present invention. The promoter and the signal peptide-encoding region of the subtilisin E gene inserted in the plasmid pKWZ as described in J. Bacteriol., 171:2657-2665 (1989) can be used. The base sequence of the 5' upstream region including the promoter sequence is described in the reference (supra) and the base sequence of the region encoding the subtilisin is described in J. Bacteriol., 158:411-418 (1984).

Based on these sequences, the primer SUB4 for

introducing an EcoRI site upstream the promoter sequence of the gene and the primer BmR1 for introducing a BamHI site downstream the region encoding the signal peptide of subtilisin E are synthesized. The base sequences of the primers SUB4 and BmR1 are shown in the SEQ ID NOS:17 and 18 of the Sequence Listing, respectively. The primers SUB4 and BmR1 can be used to amplify a DNA fragment of approximately 0.3 kb containing the promoter and the signal peptide-encoding region of the subtilisin E gene by PCR using the plasmid pKWZ as a template.

The Protease PFUS gene to be placed downstream the DNA fragment can be obtained from a chromosomal DNA from *Pyrococcus furiosus* by PCR method. The primer NPF-4 can be used as a primer that hybridizes with the 5' region of the gene. The primer NPM-1, which is designed based on the base sequence downstream from the termination codon of the gene and has a SphI site, can be used as a primer which hybridizes with the 3' region of the gene. The sequence of the primer NPM-1 is shown in the SEQ ID NO:19 of the Sequence Listing.

One BamHI site present in the gene would become a problem for a procedure in which a BamHI site is utilized for joining the Protease PFUS gene to the 0.3 kb DNA fragment. The primers mutRR and mutFR for eliminating the BamHI site by PCR-mutagenesis method can be prepared based

on the base sequence of the Protease PFUS gene as shown in the SEQ ID NO:15 of the Sequence Listing. The base sequences of the primers mutRR and mutFR are shown in the SEQ ID NOS:20 and 21 of the Sequence Listing, respectively. When these primers are used to eliminate the BamHI site, the amino acid residue encoded by this site, i.e., glycine at position 560 in the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:16 of the Sequence Listing, is substituted by valine due to the base substitution introduced into the site.

The Protease PFUS gene to be joined to the promoter and the signal peptide-encoding region of the subtilisin E gene can be obtained by using these primers. Specifically, two PCRs are performed using a chromosomal DNA from *Pyrococcus furiosus* as a template and the pair of the primers mutRR and NPF-4 or the pair of the primers mutFR and NPM-1. In addition, a second round of PCR is performed using a heteroduplex formed by mixing the respective PCR-amplified DNA fragments as a template and the primers NPF-4 and NPM-1. Thus, the entire Protease PFUS gene of approximately 2.4 kb which does not contain an internal BamHI site can be amplified.

A DNA fragment of approximately 2.4 kb obtained by digesting the PCR-amplified DNA fragment with BamHI and SphI is isolated and used to replace a BamHI-SphI fragment

in the plasmid pSNP1 which contains the Protease PFUS gene. An expression vector thus constructed is designated as the plasmid pPS1. *Bacillus subtilis* DB104 transformed with this plasmid is designated as *Bacillus subtilis* DB104/pPS1. A similar protease activity is found in either of the culture supernatant and the cell extract of the culture of this transformant as observed for the transformant harboring the plasmid pSNP1, demonstrating that the amino acid substitution does not influence the enzymatic activity. The restriction enzyme map of the plasmid pPS1 is shown in Figure 7.

The DNA fragment of approximately 0.3 kb containing the promoter and the signal peptide-encoding region of the subtilisin E gene is digested with EcoRI and BamHI and is used to replace the EcoRI-BamHI fragment containing the P43 promoter and a ribosome binding site in the plasmid pPS1. An expression plasmid thus constructed is designated as pNAPS1. *Bacillus subtilis* DB104 transformed with this plasmid is designated as *Bacillus subtilis* DB104/pNAPS1. A thermostable protease activity is found in either of the culture supernatant and the cell extract of the culture of the transformant, with the expression level being increased as compared with that of *Bacillus subtilis* DB104/pSNP1. The restriction enzyme map of the plasmid pNAPS1 is shown in Figure 8.

The protease expressed from the transformant exhibits enzymological properties equivalent to those of the protease expressed by *Bacillus subtilis* DB104/pSNP1 as described above. The protease expressed by the transformant was purified. The analysis of the N-terminal amino acid sequence of the purified protease provided the amino acid sequence as shown in the SEQ ID NO:22 of the Sequence Listing. This sequence is identical with the sequence from position 133 to position 144 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:15 of the Sequence Listing, indicating that the mature Protease PFUS is an enzyme consisting of a polypeptide starting from this portion. The amino acid sequence of the mature Protease PFUS assumed from these results is shown in the SEQ ID NO:4 of the Sequence Listing.

Although the amount of the protease produced by *Bacillus subtilis* DB104/pNAPS1 is increased as compared with the amount of the protease produced by *Bacillus subtilis* DB104/pSNP1 (FERM BP-5634), higher productivity is desired. It is expected that the expression level of the protease is increased by modifying the junction of the fused peptide encoded by pNAPS1 between the signal peptide of the subtilisin and Protease PFUS to make the removal of the signal peptide more efficient. In the plasmid pNAPS1, a peptide consisting of three amino acid residues Ala-Gly-Ser

is inserted between the C-terminal amino acid residue of the signal peptide of subtilisin E as shown in the SEQ ID NO:3 of the Sequence Listing (Ala) and the N-terminal amino acid residue of Protease PFUS (Met). A transformant with increased expression level of the protease can be obtained by introducing a mutation into the DNA encoding this peptide in the plasmid pNAPS1 and examining the protease productivity of the transformant into which the mutant plasmid is introduced.

First, a mutant plasmid is prepared in which the portion encoding Ser in the three amino acid peptide in the gene encoding the fused protein, subtilisin E-Protease PFUS, in the plasmid pNAPS1 is modified such that the base sequence of the portion encodes random two amino acid residues. Such a mutant plasmid can be created by means of PCR. For example, the primers SPOF0 and SPOR0 having sequences in which the codon encoding Ser (TCC) is substituted by random six bases (the base sequences of the primers SPOF0 and SPOR0 are shown in the SEQ ID NOS:24 and 25 of the Sequence Listing, respectively) and the primers SUB3 and NPR-10 which are prepared based on the base sequence around this region (the base sequences of the primers SUB3 and NPR-10 are shown in the SEQ ID NOS:26 and 27 of the Sequence Listing, respectively) can be used to perform PCR to obtain a DNA fragment into which the

intended mutation at the portion corresponding to the codon encoding Ser (TCC) is introduced. A mutant plasmid containing the protease gene with the introduced mutation can be obtained by replacing the resulting fragment for the corresponding region in the plasmid pNAPS1.

A transformant with increased expression level can be then obtained by introducing the mutant plasmids thus obtained into an appropriate host, for example, *Bacillus subtilis* DB104, and determining the level of the protease expressed by the transformants. The expression level of the protease can be confirmed by determining the activity in the independent culture of the isolated transformant. Alternatively, a transformant with increased expression level can be readily selected by using an agar plate containing a substrate.

Specifically, the transformants into which the mutant plasmids are introduced are grown on agar plates containing skim milk. Thereafter, the plates are incubated at temperature at which Protease PFUS exhibits its activity, for example, at 70°C. Skim milk around a colony of a transformant expressing a protease is degraded to become clear. The expression level of the protease can be estimated from the size of the clear zone.

One of the transformants thus obtained which express high level of protease activity as compared with

Bacillus subtilis DB104/pNAPS1 is designated as *Bacillus subtilis* DB104/pSPO124. The plasmid contained in this transformant was prepared (this plasmid is designated as pSPO124). Analysis of the base sequence of the plasmid revealed that the portion encoding Ser was changed into a base sequence GGGAAT, that is, that a protein in which Ser was changed into Gly-Asn was encoded by the plasmid.

Thus, it proved that the expression level of the protein of interest can be increased in a bacterium of genus *Bacillus* as a host by placing a peptide consisting of four amino acid residues Ala-Gly-Gly-Asn downstream the signal peptide of a subtilisin, fusing it to the N-terminus of the protein of interest and expressing the fused protein. In addition to subtilisin E (from *Bacillus subtilis*) which is used in the present invention, subtilisin BPN' from *Bacillus amyloliquefaciens* (Nucl. Acids Res., 11:7911-7925 (1983)), subtilisin Carlsberg from *Bacillus licheniformis* (Nucl. Acids Res., 13:8913-8926 (1985)) and the like are known as subtilisins produced by bacteria of genus *Bacillus*. The signal peptides from them can be preferably used for the present invention although their amino acid sequences slightly vary each other. Various promoters which function in a bacterium of genus *Bacillus* can be used in place of the promoter from the subtilisin E gene which is used in the present invention for controlling expression.

There is no limitation regarding the protein to be expressed. It is possible to express a protein at high level by genetic engineering technique by applying the present invention as long as the gene for the protein is available. It is evident that the present invention can be utilized to express a protein derived from an organism other than the host from the fact that a protein derived from *Pyrococcus furiosus*, which is taxonomically different from bacteria of genus *Bacillus*, is expressed at high level. The present invention is preferably used to produce Protease PFUL, Protease TCES as well as proteases from *Staphylothermus marinus* and *Thermobacteroides proteoliticus* that are structurally similar to Protease PFUS by genetic engineering technique.

Based on the homology with subtilisins, it is considered that Protease PFUS is expressed as a precursor protein having a signal peptide and a propeptide and then subjected to processing to generate a mature enzyme. Furthermore, based on the results of the N-terminal amino acid sequence analysis of the mature Protease PFUS enzyme, it may be assumed that the mature enzyme is an enzyme consisting of the amino acid sequence as shown in the SEQ ID NO:4 of the Sequence Listing. However, the molecular weight of the purified mature Protease PFUS is approximately 45 kDa which is smaller than that calculated

from the amino acid sequence, suggesting that Protease PFUS expressed as a precursor is converted to a mature protease after being subjected to processing of its C-terminal peptide as well.

If the C-terminal peptide removed by the processing is not essential to the enzymatic activity or the folding of the enzyme protein into proper structure, it is expected that the expression level of Protease PFUS can be also increased by deleting the region encoding this portion from the gene and expressing the protease.

The molecular weight of the mature Protease PFUS obtained from *Bacillus subtilis* DB104/pNAPS1 can be precisely measured, for example, by using a mass spectrometer. It is found from the measured molecular weight and the N-terminal amino acid sequence of the mature Protease PFUS determined as described above that the protease is a polypeptide corresponding to Ala at position 133 to Thr at position 552 of the amino acid sequence as shown in the SEQ ID NO:15 of the Sequence Listing. Furthermore, a plasmid which expresses Protease PFUS lacking a polypeptide nonessential for its enzymatic activity can be constructed by introducing a termination codon in the vicinity of the portion encoding Thr at position 552 in the Protease PFUS gene contained in the plasmid pNAPS1. Specifically, a DNA fragment having a base

sequence into which the intended termination codon is introduced can be obtained by PCR using the primer NPR544 which can introduce a termination codon (TGA) on the C-terminal side of the 544th amino acid residue encoding codon from the initiation codon in the Protease PFUS gene in the plasmid pNAPS1 (Ser) (the base sequence of the primer NPR544 is shown in the SEQ ID NO:28 of the Sequence Listing) and the primer NPFE81 which has the base sequence of the region upstream from the NspV site in the gene (the base sequence of the primer NPFE81 is shown in the SEQ ID NO:29 of the Sequence Listing). A mutant plasmid containing the protease gene into which the mutation of interest is introduced can be then obtained by replacing the fragment for the corresponding region in the plasmid pNAPS1. This plasmid is designated as the plasmid pNAPSΔC. *Bacillus subtilis* DB104 transformed with this plasmid is designated as *Bacillus subtilis* DB104/pNAPSΔC.

This transformant expresses a protease activity having properties equivalent to those of Protease PFUS, with the expression level being higher than that of *Bacillus subtilis* DB104/pNAPS1.

Thus, it was found that the Protease PFUS gene contained in the plasmid pNAPSΔC has a sufficient region to express the activity of the enzyme. The base sequence of the region encoding Protease PFUS present in the plasmid is

shown in the SEQ ID NO:2 of the Sequence Listing. The amino acid sequence encoded by the base sequence is shown in the SEQ ID NO:1 of the Sequence Listing.

Furthermore, Protease PFUS lacking its C-terminal peptide can be expressed by introducing a mutation similar to that in the plasmid pNAPSΔC into the Protease PFUS gene in the plasmid pSPO124.

Specifically, the plasmid of interest can be constructed by mixing and ligating a DNA fragment of approximately 13 kb obtained by digesting the plasmid pNAPSΔC with NspV and SphI with the plasmid pSPO124 that has been digested with NspV and SphI. This plasmid is designated as the plasmid pSO124ΔC. *Bacillus subtilis* DB104 transformed with this plasmid is designated and indicated as *Bacillus subtilis* DB104/pSO124ΔC, and deposited on May 16, 1997 (the date of the original deposit) under Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan under accession number FERM BP-6294. The expression level of protease of this transformant is increased as compared with that of *Bacillus subtilis* DB104/pNAPS1.

The enzymological properties as well as the physical and chemical properties of the proteases produced

by the transformants, *Bacillus subtilis* DB104/pNAPSΔC and *Bacillus subtilis* DB104/pSPO124ΔC appear to be identical with those of the protease produced by *Bacillus subtilis* DB104/pSNP1. The main properties of the proteases obtained from the cultures of the two transformants are as follows:

(1) Action:

Degrades casein and gelatin to generate short chain polypeptides.

Hydrolyzes succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) to generate a fluorescent substance (7-amino-4-methylcoumarin).

Hydrolyzes succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) to generate a yellow substance (p-nitroaniline).

(2) Optimal temperature:

Exhibits an enzymatic activity at 40-110°C, with the optimal temperature being 80-95°C.

(3) Optimal pH:

Exhibits an enzymatic activity at pH 5-10, with the optimal pH being pH 6-8.

(4) Thermostability:

Retains 90% or more of its enzymatic activity after treatment at 95°C for 8 hours.

(5) pH stability

Retains 95% or more of its activity after treatment at pH 5-11 at 95°C for 60 minutes.

(6) Molecular weight

Exhibits a molecular weight of approximately 45 kDa on SDS-PAGE.

Thus, proteases having high thermostability and genes therefor are provided. Also, a novel system for expressing a protein, which enables the expression of the protease in large quantity is disclosed by the present invention. The expression system is useful in production of the protease of the present invention as well as various proteins by genetic engineering technique.

The following Examples illustrate the present invention in more detail, but are not to be construed to limit the scope thereof.

Example 1

(1) Preparation of a chromosomal DNA from *Pyrococcus furiosus*

Pyrococcus furiosus DSM3638 was cultured as follows.

A medium containing 1% Tryptone, 0.5% yeast extract, 1% soluble starch, 3.5% Jamarine S Solid (Jamarine Laboratory), 0.5% Jamarine S Liquid (Jamarine Laboratory), 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CoSO_4$,

0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm H_3BO_3, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$, 0.25 ppm $NiCl_2 \cdot H_2O$ was placed in a 2 L medium bottle, sterilized at 120°C for 20 minutes, bubbled with nitrogen gas to remove dissolved oxygen, then the strain was inoculated into the medium and cultured at 95°C for 16 hours without shaking. After cultivation, cells were collected by centrifugation.

The resulting cells were then suspended in 4 mL of 50 mM Tris-HCl (pH 8.0) containing 25% sucrose. 2 mL of 0.2 M EDTA and 0.8 mL of lysozyme (5 mg/mL) were added to the suspension. The mixture was incubated at 20°C for 1 hour. 24 mL of SET solution (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0), 4 mL of 5% SDS and 400 µL of proteinase K (10 mg/mL) were then added to the mixture. Incubation was further carried out at 37°C for 1 hour. The reaction was terminated by extracting the mixture with phenol-chloroform. Then, ethanol precipitation was carried out to obtain approximately 3.2mg of chromosomal DNA.

Example 2

(1) Synthesis of primers for constructing the plasmid pNSP1

In order to synthesize primers used to amplify the entire Protease PFUS gene, the plasmid pSNP1 that contains the entire gene was isolated from *Bacillus*

subtilis DB104/pSNP1 (FERM BP-5634) and the base sequence of the required region was determined. Based on the base sequence, the primer NPF-4 for introducing a BamHI site immediately upstream the initiation codon of the Protease PFUS gene and the primer NPM-1 which hybridizes with the 3' region of the gene and contains a recognition site for SphI were synthesized. The base sequences of the primers NPF-4 and NPM-1 are shown in the SEQ ID NOS:13 and 19 of the Sequence Listing, respectively.

The primers mutRR and mutFR for removing the BamHI site present approximately 1.7 kb downstream from the initiation codon in the Protease PFUS gene were also synthesized. The base sequences of the primers mutRR and mutFR are shown in the SEQ ID NOS:20 and 21 of the Sequence Listing, respectively.

(2) Preparation of the plasmid pPS1

Two sets of LA-PCR reaction mixtures each of which containing a chromosomal DNA from *Pyrococcus furiosus* as a template and a combination of the primers NPF-4 and mutRR or a combination of the primers mutFR and NPM-1 were prepared and subjected to 30 cycles of reactions of 94°C for 30 seconds-55°C for 1 minute-68°C for 3 minutes. LA PCR Kit Ver. 2 (Takara Shuzo) was used to prepare the LA-PCR reaction mixtures. Aliquots of the reaction mixtures were subjected to agarose gel electrophoresis, and amplification

of a DNA fragment of approximately 1.8 kb with the primers NPF-4 and mutRR and a DNA fragment of approximately 0.6 kb with the primers mutFR and NPM-1 were observed, respectively.

The primers were removed from the two PCR reaction mixtures using SUPREC-02 (Takara Shuzo) to prepare amplified DNA fragments. An LA-PCR reaction mixture which contained these two amplified DNA fragments and did not contain the primers or LA Taq was prepared, heat-denatured at 94°C for 10 minutes, cooled to 30°C within 30 minutes, then incubated at 30°C for 15 minutes to form a heteroduplex. Subsequently, LA Taq (Takara Shuzo) was added to the reaction mixture to react at 72°C for 30 minutes. The primers NPF-4 and NPM-1 were then added to the reaction mixture, which was then subjected to 25 cycles of reactions of 94°C for 30 seconds-55°C for 1 minute-68°C for 3 minutes. Amplification of a DNA fragment of approximately 2.4 kb was observed in the reaction mixture.

The DNA fragment of approximately 2.4 kb was digested with BamHI and SphI (both from Takara Shuzo). The fragment was mixed and ligated with the plasmid pSNP1 which had been digested with BamHI and SphI to remove the entire Protease PFUS gene, then introduced into *Bacillus subtilis* DB104. Plasmids were prepared from resulting kanamycin-resistant transformants, and a plasmid into which only one

molecule of the fragment of approximately 2.4 kb was inserted was selected and designated as the plasmid pPS1. *Bacillus subtilis* DB104 transformed with this plasmid pPS1 was designated as *Bacillus subtilis* DB104/pPS1.

The restriction enzyme map of the plasmid pPS1 is shown in Figure 7.

(3) Amplification of a DNA fragment for the promoter-signal peptide-encoding region of the subtilisin E gene

Primers for obtaining the promoter-signal peptide-encoding region of the subtilisin E gene were synthesized. First, the primer SUB4 was synthesized based on the base sequence of the promoter region of the subtilisin E gene as described in J. Bacteriol., 171:2657-2665 (1989), which hybridizes with the sequence upstream this region and contains an EcoRI site (the base sequence of the primer SUB4 is shown in the SEQ ID NO:17 of the Sequence Listing). The primer BmR1 which is capable of introducing a BamHI site immediately downstream the signal peptide-encoding region was synthesized based on the base sequence of the subtilisin E gene as described in J. Bacteriol., 158:411-418 (1984) (the base sequence of the primer BmR1 is shown in the SEQ ID NO:18 of the Sequence Listing).

A PCR reaction mixture containing the plasmid

pKWZ, which contains the subtilisin E gene as described in J. Bacteriol., 171:2657-2665, as a template and the primers SUB4 and BmR1 was prepared and subjected to 30 cycles of reactions of 94°C for 30 seconds-55°C for 1 munute-68°C for 2 minutes. An aliquot of the reaction mixture was subjected to agarose gel electrophoresis, and amplification of a DNA fragment of approximately 0.3 kb was observed.

(4) Construction of the protease expression plasmid pNAPS1

The DNA fragment of approximately 0.3 kb as described above was digested with EcoRI (Takara Shuzo) and BamHI, mixed and ligeted with the plasmid pPS1 described in Example 3 which had been digested with EcoRI and BamHI, then introduced into *Bacillus subtilis* DB104. Plasmids were prepared from resulting kanamycin-resistant transformants, and a plasmid into which only one molecule of the fragment of approximately 0.3 kb was inserted was selected and designated as the plasmid pNAPS1. *Bacillus subtilis* DB104 transformed with the plasmid pNAPS1 was designated as *Bacillus subtilis* DB104/pNAPS1.

The restriction enzyme map of the plasmid pNAPS1 is shown in Figure 8.

(5) Construction of the plasmid pSNP2

The primer SUB17R for introducing a BamHI site upstream the signal peptide-encoding region of the

subtilisin E gene in the above-mentioned plasmid pNAPS1 was synthesized (the base sequence of the primer SUB17R is shown in the SEQ ID NO:23 of the Sequence Listing). A PCR reaction mixture containing the plasmid pNAPS1 as a template and the primers SUB17R and SUB4 was prepared and subjected to 25 cycles of reactions of 94°C for 30 seconds-55°C for 1 munute-72°C for 1 minute. The amplified DNA fragment of approximately 0.21 kb was digested with EcoRI and BamHI to obtain a DNA fragment of approximately 0.2 kb that contains the promoter and the SD sequence of the subtilisin E gene. This fragment was mixed and ligated with the plasmid pNAPS1 that had been digested with EcoRI and BamHI. The reaction mixture was used to transform *Bacillus subtilis* DB104. Plasmids were prepared from resulting kanamycin-resistant transformants, and a plasmid into which the DNA fragment of approximately 0.2 kb was inserted was selected and designated as the plasmid pSNP2.

(6) Generation of a mutant plasmid which expresses a protease at high level

The primers SPOF0 and SPOR0 for substituting the sequence encoding the amino acid residue Ser (base sequence: TCC) at the junction between the signal peptide-encoding region of the subtilisin E gene in the plasmid pNAPS1 and the initiation codon of the Protease PFUS gene with a sequence for two random amino acid residues were

synthesized (the base sequences of the primers SPOF0 and SPOR0 are shown in the SEQ ID NOS:24 and 25 of the Sequence Listing, respectively). The primer SUB3 for introducing a BamHI site immediately upstream the signal peptide-encoding region in the subtilisin E gene in the plasmid pNAPS1 and the primer NPR-10 which contains a SpeI site within the Protease PFUS encoding region were synthesized (the base sequences of the primers SUB3 and NPR-10 are shown in the SEQ ID NOS:26 and 27 of the Sequence Listing, respectively).

PCR reaction mixtures each of which containing the plasmid pNAPS1 as a template and a combination of the primers SPOF0 and NPR-10 or a combination of the primers SUB3 and SPOR0 were prepared and subjected to 20 cycles of reactions of 94°C for 30 seconds-50°C for 1 munute-72°C for 1 minute. DNA fragments of approximately 0.13 kb and approximately 0.35 kb amplified in the two reaction mixtures were mixed together, denatured at 94°C for 10 minutes, cooled gradually to 37°C to form a heteroduplex. A double-stranded DNA was then generated from the heteroduplex by means of Taq polymerase (Takara Shuzo). A PCR reaction mixture containing the double-stranded DNA thus obtained as a template and the primers SUB3 and NPR-10 was prepared and subjected to 25 cycles of reactions of 94°C for 30 seconds-50°C for 1 minute-72°C for 1 minute. A DNA fragment obtained by digesting the amplified DNA

fragment of approximately 0.43 kb with BamHI and SpeI (Takara Shuzo) was mixed and ligated with the plasmid pSNP2 that had been digested with BamHI and SpeI. The reaction mixture was used to transform *Bacillus subtilis* DB104.

Resulting kanamycin-resistant transformants were inoculated on skim milk plates (LB-agar medium for high temperature cultivation containing 10 μg/mL of kanamycin and 1% skim milk) to form colonies. Subsequently, the plates were incubated at 70°C and the protease activities expressed by the respective transformants were examined based on the degree of degradation of the skim milk around the colonies. As a result, one clone that exhibited a particularly high activity was isolated and a plasmid, which was designated as the plasmid pSPO124, was prepared from the clone. *Bacillus subtilis* DB104 transformed with this plasmid was designated as *Bacillus subtilis* DB104/pSPO124. The base sequence of the plasmid pSPO124 was analyzed, and it was found that the base sequence which encodes Ser in the plasmid pNAPS1 was substituted by a base sequence GGGAAT, that is, that a protein in which Ser was changed to two amino acid residues Gly-Asn was encoded. Additionally, it proved that the 25th codon from the initiation codon corresponding to Pro (CCA) of the Protease PFUS gene was changed to a codon encoding Leu (CTA) simultaneously with the mutation as described above.

(7) Construction of the protease expression plasmid pNAPSΔC

A termination codon was introduced on the C-terminal side of the 544th amino acid residue from the initiation codon of the Protease PFUS gene in the plasmid pNAPS1 to construct a plasmid which expresses a protease lacking downstream from this site. The primer NPR544 which introduces a termination codon (base sequence: TGA) on the C-terminal side of the codon encoding the 544th amino acid residue in the gene and has an SphI site was synthesized (the base sequence of the primer NPR544 is shown in the SEQ ID NO:28 of the Sequence Listing). In addition, the primer NPFE81 was synthesized based on the base sequence of the portion upstream from the NspV site in the gene (the base sequence of the primer NPFE81 is shown in the SEQ ID NO:29 of the Sequence Listing).

A PCR reaction mixture containing the plasmid pNAPS1 as a template and the primers NPFE81 and NPR544 was prepared and subjected to 20 cycles of reactions of 94°C for 30 seconds-50°C for 1 minute-72°C for 1 minute. The amplified DNA fragment of approximately 0.61 kb was digested with NspV (Takara Shuzo) and SpeI to obtain a DNA fragment of approximately 0.13 kb containing the termination codon. This DNA fragment was mixed and ligated with the plasmid pNAPS1 that had been digested with

restriction enzymes NspV and SphI. The reaction mixture was used to transform *Bacillus subtilis* DB104. Plasmids were prepared from the resulting kanamycin-resistant transformants, a plasmid into which the DNA fragment of approximately 0.13 kb was inserted was selected and designated as the plasmid pNAPSΔC. *Bacillus subtilis* DB104 transformed with the plasmid pNAPSΔC was designated as *Bacillus subtilis* DB104/pNAPSΔC.

(8) Construction of the protease expression plasmid pSPO124ΔC

A DNA fragment of approximately 1.3 kb obtained by digesting the plasmid pNAPSΔC with NspV and SphI was isolated, then mixed and ligated with the plasmid pSPO124 that had been digested with NspV and SphI. The reaction mixture was used to transform *Bacillus subtilis* DB104. Plasmids were prepared from the resulting kanamycin-resistant transformants, a plasmid into which the DNA fragment of approximately 1.3 kb was inserted was selected and designated as the plasmid pSPO124ΔC. *Bacillus subtilis* DB104 transformed with the plasmid pSPO124ΔC was designated as *Bacillus subtilis* DB104/pSPO124ΔC.

Example 3

(1) Cultivation of *Bacillus subtilis* transformed with a plasmid containing the Protease PFUS gene and

preparation of a crude enzyme solution

Bacillus subtilis DB104/pNAPS1, which is *Bacillus subtilis* DB104 into which the plasmid pNAPS1 containing the Protease PFUS gene was introduced as described in Example 2, was cultured in 2 mL of LB medium (Tryptone 10 g/L, yeast extract 5g/L, NaCl 5g/L, pH 7.2) containing 10 μg/mL of kanamycin at 37°C for 24 hours. The culture was centrifuged to obtain a culture supernatant (the preparation 1-S) and cells.

The cells were suspended in 100 μL of 50 mM Tris-HCl, pH 7.5 and digested at 37°C for 45 minutes after an addition of 2 mg of lysozyme (Sigma). The digested sample was heat-treated at 95°C for 10 minutes, and then a supernatant was collected by centrifugation to obtain a cell-free extract (the preparation 1-L).

Similarly, culture supernatants and cell-free extracts were obtained from *Bacillus subtilis* DB104/pSPO124 containing the plasmid pSPO124, *Bacillus subtilis* DB104/pNAPSΔC containing the plasmid pNAPSΔC or *Bacillus subtilis* DB104/pSPO124ΔC containing the plasmid pSPO124ΔC. The culture supernatant and the cell-free extract from *Bacillus subtilis* DB104/pSPO124 were designated as 124-S and 124-L, respectively. The culture supernatant and the cell-free extract from *Bacillus subtilis* DB104/pNAPSΔC were designated as ΔC-S and ΔC-L, respectively. The culture

supernatant and the cell-free extract from *Bacillus subtilis* DB104/pSPO124ΔC were designated as 124ΔC-S and 124ΔC-L, respectively. Protease activities were determined with these preparations and the concentration of the protease contained in each preparation was determined.

(2) Comparison of protease productivities

The activity of Protease PFUS was determined by spectroscopically measuring the amount of p-nitroaniline generated in an enzymatic hydrolysis reaction using Suc-Ala-Ala-Pro-Phe-p-NA (Sigma) as a substrate. Briefly, an enzyme preparation to be measured for its enzymatic activity was appropriately diluted. 50 µL of 1 mM Suc-Ala-Ala-Pro-Phe-p-NA solution in 100 mM phosphate buffer, pH 7.0 was added to 50 µL of the diluted sample solution. Then, the reaction was allowed to proceed at 95°C for 30 minutes. After terminating the reaction by cooling on ice, absorbance at 405 nm was measured to calculate the amount of p-nitroaniline generated. One unit of the enzyme was defined as the amount of the enzyme which generated 1 µmole of p-nitroaniline per 1 minute at 95°C. The amount of enzyme protein expressed in the culture supernatant or the cells was calculated based on the measured enzymatic activity assuming the specific activity as 9.5 unit/mg protein of Protease PFUS.

The protease activity of each enzyme preparation

prepared in Example 3-(1) was measured. The productivity of Protease PFUS per 1 L of culture of each transformant calculated from the measurement is shown in Table 1.

In *Bacillus subtilis* DB104/pSPO124, the productivity of Protease PFUS in the cells increased by 3.6 fold as compared with that of *Bacillus subtilis* DB104/pNAPS1. In *Bacillus subtilis* DB104/pNAPSΔC, the productivity of Protease PFUS increased in the culture supernatant by 2.4 fold and in the cells by 2.2 fold, respectively. Also, in *Bacillus subtilis* DB104/pSPO124ΔC, the productivity of Protease PFUS increased in the culture supernatant by 2 fold and in the cells by 2.4 fold, respectively. The productivity per cells also increased.

The total amount of Protease PFUS produced in the culture supernatant and the cells increased by 2.1 fold for *Bacillus subtilis* DB104/pSPO124, by 2.1 fold for *Bacillus subtilis* DB104/pNAPSΔC and by 2.2 fold for *Bacillus subtilis* DB104/pSPO124ΔC, respectively, as compared with that of *Bacillus subtilis* DB104/pNAPS1.

Table 1

The productivity of Protease PFUS (mg/L of culture)

Transformant (Plasmid)	Culture Supernatant	Cells	Culture Supernatant + Cells
pNAPS1	15.1	12.5	27.6
pSPO124	13.1	45.4	58.5
pNAPSΔC	35.5	28.1	63.6
pSPO124ΔC	30.5	30.1	60.6

Example 4

(1) Preparation of purified enzyme preparation of the mature Protease PFUS

Bacillus subtilis DB104/pNAPS1 and *Bacillus subtilis* DB104/pSPO124ΔC, both of which are *Bacillus subtilis* DB104 into which the gene for the hyperthermostable protease of the present invention was introduced as described in Example 2, were separately inoculated into 5 mL of LB medium containing 10 μg/mL kanamycin and cultured with shaking at 37°C for 7 hours. The cultures of 5 mL were inoculated into 500 mL of TM medium (soybean powder 5 g/L, Polypeptone 10 g/L, meat extract 5 g/L, yeast extract 2 g/L, glucose 10 g/L, $FeSO_4 \cdot 7H_2O$ 10 mg/L, $MnSO_4 \cdot 4H_2O$ 10 mg/L, $ZnSO_4 \cdot 7H_2O$ 1 mg/L, pH 7.0) containing 10 μg/mL of kanamycin in 5 L Erlenmeyer flasks and cultured with shaking at 30°C for 3 days. The resulting cultures were sonicated, heat-treated at 95°C for 30 minutes, then centrifuged to collect supernatants.

Ammonium sulfate was added to the supernatants to 25% saturation, then the supernatants obtained by subsequent centrifugation were applied to Micro-Prep Methyl HIC columns (Bio-Rad) equilibrated with 25 mM Tris-HCl buffer (pH 7.6) containing 25% saturated ammonium sulfate. After washing the gel with the same buffer, Protease PFUS adsorbed to the columns was eluted by stepwise elution using 25 mM Tris-HCl buffer (pH 7.6) containing 40% ethanol. The fractions containing Protease PFUS thus obtained were subjected to gel filtration using NAP-25 columns (Pharmacia) equilibrated with 0.05% trifluoroacetic acid containing 20% acetonitrile, desalted while denaturing Protease PFUS, then purified preparations of Protease PFUS were obtained. The preparations obtained from *Bacillus subtilis* DB104/pNAPS1 and *Bacillus subtilis* DB104/pSPO124ΔC were designated as NAPS-1 and SPO-124ΔC, respectively.

Electrophoresis of both of the purified enzyme preparations on 0.1% SDS-10% polyacrylamide gel followed by staining with Coomassie Brilliant Blue R-250 revealed single bands for both of the purified enzyme preparations NAPS-1 and SPO-124ΔC with an estimated molecular weight of approximately 45 kDa.

(2) Analysis of the N-terminal amino acid sequence of the mature Protease PFUS

N-terminal amino acid sequences of the purified

enzyme preparations NAPS-1 and SPO-124ΔC were analyzed by automated Edman method using G1000A protein sequencer (Hewlett-Packard). Both of the N-terminal amino acid sequences of the two purified enzyme preparations were as shown in the SEQ ID NO:22 of the Sequence Listing. This sequence coincides with the sequence from position 133 to position 144 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:15 of the Sequence Listing, indicating that both of NAPS-1 and SPO-124ΔC are enzymes consisting of a polypeptide starting from this portion.

(3) Mass spectrometric analysis of the mature Protease PFUS

Mass spectrometric analysis on the purified enzyme preparations NAPS-1 and SPO-124ΔC was carried out using API300 quadrupole triple mass spectrometer (Perkin-Elmer Sciex). Based on the estimated molecular weight of NAPS-1, 43,744 Da, it was demonstrated that the mature Protease PFUS produced by *Bacillus subtilis* DB104/pNAPS1 is an enzyme consisting of a polypeptide from Ala at position 133 to Thr at position 552 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:15 of the Sequence Listing. Furthermore, based on the estimated molecular weight of SPO-124ΔC, 42,906 Da, it was demonstrated that the mature Protease PFUS produced by *Bacillus subtilis* DB104/pSPO124ΔC is an enzyme consisting of a polypeptide

from Ala at position 133 to Ser at position 544 of the amino acid sequence of Protease PFUS as shown in the SEQ ID NO:15 of the Sequence Listing, i.e., the amino acid sequence as shown in the SEQ ID NO:2 of the Sequence Listing.

10095924.030902